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Abstract: Lipins are eukaryotic proteins with functions in lipid synthesis and the homeostatic control of energy balance. They execute these functions by acting as phosphatidate phosphatase enzymes in the cytoplasm and by changing gene expression after translocation into the cell nucleus, in particular under fasting conditions. Here, we asked whether nuclear translocation and the enzymatic activity of Drosophila Lipin serve essential functions and how gene expression changes, under both fed and fasting conditions, when nuclear translocation is impaired. To address these questions, we created a Lipin null mutant, a mutant expressing Lipin lacking a nuclear localization signal (LipinΔNLS), and a mutant expressing enzymatically dead Lipin. Our data support the conclusion that the enzymatic but not nuclear gene regulatory activity of Lipin is essential for survival. Notably, adult LipinΔNLS flies were not only viable but also exhibited improved life expectancy. In contrast, they were highly susceptible to starvation. Both the improved life expectancy in the fed state and the decreased survival in the fasting state correlated with changes in metabolic gene expression. Moreover, increased life expectancy of fed flies was associated with a decreased metabolic rate. Interestingly, in addition to metabolic genes, genes involved in feeding behavior and the immune response were misregulated in LipinΔNLS flies. Altogether, our data suggest that the nuclear activity of Lipin influences the genomic response to nutrient availability with effects on life expectancy and starvation resistance. Thus, nutritional or therapeutic approaches that aim at lowering nuclear translocation of lipins in humans may be worth exploring.

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Nuclear translocation ability of Lipin differentially affects gene expression and survival in fed and fasting *Drosophila*

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Running title: Biological significance of Lipin nuclear translocation

Abbreviations:

NLS, Nuclear Localization Signal; PAP, phosphatidic acid phosphatase; TAG, triacylglycerol; TOR, target of rapamycin

ABSTRACT

Lipins are eukaryotic proteins with functions in lipid synthesis and the homeostatic control of energy balance. They execute these functions by acting as phosphatidate phosphatase enzymes in the cytoplasm and by changing gene expression after translocation into the cell nucleus, in particular under fasting conditions. Here, we asked whether nuclear translocation and enzymatic activity of *Drosophila* Lipin serve essential functions and how gene expression changes, under both fed and fasting conditions, when nuclear translocation is impaired. To address these questions, we created a *Lipin* null mutant, a mutant expressing Lipin lacking a nuclear localization signal ($Lipin^{\Delta NLS}$), and a mutant expressing enzymatically dead Lipin. Our data support the conclusion that the enzymatic, but not the nuclear gene regulatory activity of Lipin, is essential for survival. Notably, adult $Lipin^{\Delta NLS}$ flies were not only viable but also exhibited improved life expectancy. In contrast, they were highly susceptible to starvation. Both the improved life expectancy in the fed state and the decreased survival in the fasting state correlated with changes in metabolic gene expression. Moreover, increased life expectancy of fed flies was associated with a decreased metabolic rate. Interestingly, in addition to metabolic genes, genes involved in feeding behavior and the immune response were mis-regulated in $Lipin^{\Delta NLS}$ flies. Combined, our data suggest that nuclear activity of Lipin influences the genomic response to nutrient availability with effects on life expectancy and starvation resistance. Thus, nutritional or therapeutic approaches that aim at lowering nuclear translocation of lipins in humans may be worth exploring.

KEYWORDS

Lipin; nuclear translocation; genomic starvation response; energy metabolism; metabolic health; fasting; feeding behavior; immune response; *Drosophila*

INTRODUCTION

Organisms adjust their metabolism to nutrient availability and, when nutrients become scarce, rely on energy stores in the form of glycogen and triacylglycerols (TAG). Adjustments require regulation of the balance between the synthesis and breakdown of storage molecules and are associated with changes in feeding behavior (1, 2). A linchpin in this regulatory system is provided by lipins, a group of proteins with dual functions in lipid synthesis and the genomic control of energy metabolism. Lipins, which are conserved among all eukaryotes, can convert phosphatidic acid into diacylglycerol as phosphatidate phosphatases (PAP) (3-5). This activity is required for TAG synthesis and the synthesis of membrane phospholipids (6). In addition, lipins can translocate into the cell nucleus to participate in gene regulation (7-11). Nuclear translocation of both mammalian lipin 1 and the single Lipin ortholog of *Drosophila* occurs under fasting conditions and is controlled by the nutrient-sensitive target of rapamycin complex 1 (TORC1) pathway (12, 13).

While the mechanism by which lipins participate in gene regulation has been explored to some extent, little is known about the genomic changes that depend on the nuclear translocation of lipins. ChIP experiments indicate that the yeast lipin ortholog Smp2 and mammalian lipin 1 associate with the promoter regions of specific genes, suggesting a function as transcriptional co-regulators (14, 15). However, at least some of the genomic effects of mouse lipin 1 depend on its PAP activity and the effect of the protein on nuclear abundance of the transcription factor SREBP1 (13). Genes responding to lipins

have been identified through an overexpression approach in mice (15), in relation to glucose feeding in *C. elegans* (16), and through a candidate gene approach in yeast (14).

Similar to mice lacking lipin 1 (4), *Drosophila* larvae that express reduced amounts of Lipin exhibit a severe underdevelopment of the fat tissue and reduced TAG stores. However, *Drosophila Lipin* is also broadly expressed outside the fat body, the insect equivalent of vertebrate adipose tissue (17). While the lack of energy reserves that accompanies the loss of fat body prevents successful metamorphosis of *Lipin* mutant larvae into adult flies, it is unknown if functions of *Lipin* in other tissues are equally required for survival and the development of adult flies. Here, we address this question by rescue experiments using a *Lipin* null mutant. Furthermore, we ask if a functional PAP motif of Lipin and the nuclear translocation signal (NLS) are essential for survival under fed and fasting conditions. Successful creation of a CRISPR/Cas9-generated *Lipin* mutant without the NLS allowed us to address how interference with nuclear translocation affects gene expression under both fed and fasting conditions. Our data show that Lipin and its PAP activity both inside and outside the fat body are essential for survival, whereas the NLS appears non-essential. Interference with nuclear translocation of the protein under both fed or fasting conditions leads to substantial changes in the expression of genes involved in energy homeostasis and feeding behavior, and of genes involved in the immune response in the fasting state. Notably, as long as sufficient nutrients are available, nuclear functions of Lipin are not only dispensable, but interference with nuclear entry of Lipin is even beneficial for survival. This observation suggests that a better understanding of nutrients that influence nuclear

entry of lipins may shed light on unhealthy, life-shortening dietary conditions. Ultimately, nuclear translocation may prove to be a property of lipins that can be utilized as a target for specific therapeutic interventions. In summary, our data substantiate the critical role that Lipin has in metabolic adaptation to starvation and they identify gene-regulatory functions in the fed state that have an effect on health and life expectancy.

MATERIALS AND METHODS

Fly stocks

Flies carrying *r*^{4}*-gal4* were provided by Jae Park (18), and flies carrying *Df(2R)Exel7095* (#7860), *tub-GAL4* (#5138), or *UAS-2xeGFP* (#6874) were obtained from the Bloomington Drosophila Stock Center. Generation of a *UAS-LipinWT* transgenic fly line has been described before (12).

Mutagenesis

CRISPR/Cas9 mutagenesis was used to create an in-frame deletion of the 18-bp DNA segment 5'-AAGAAGCGGCGCAAGAAG-3', which encodes the NLS of the Lipin protein (amino acid positions 276-281). For introduction of the NLS deletion, we used as a repair template a single-stranded oligonucleotide of the sequence 5'-GCGTCTCCGCCGAAGGCAAATCACCGCCGCCGGCGCTGCCCAATGAGCTGCTTGAAGAGTTCTTGCGCTGGGCATTCTTCTTCATTTGCGAGGTTTTGCTCTTGGACACCTCCTTGGTGGCTTCGCT -3' (Integrated DNA Technologies). This oligonucleotide corresponds to lower-strand genomic DNA extending 36 bp upstream and 91 bp downstream of the NLS but lacks the NLS itself. To construct the vector for Cas9 guide RNA expression, a protospacer of the sequence 5' -CGACTTCTTGCGCCGCTTCT-3' was cloned into pCFD3 (Addgene). Guide RNA expression vector and mutagenic repair template were injected into embryos expressing Cas9 in the germline (Bestgene Inc.;

Bloomington injection stock #54591, y[1] M{w[+mC]=nos-Cas9.P}ZH-2A w[*]). Balanced stocks were established for individual mutagenized chromosomes and screened by PCR and DNA sequencing for the presence of the NLS deletion using forward and reverse primers of the sequences 5'-CGCTGGACAACCAAAGCAAA-3' and 5'-CTCCGTGTCGCTGAAGAAGT-3', respectively.

The *Lipin*^{KO} mutant was created by CRISPR/Cas9 mutagenesis using a guide RNA targeting the region immediately downstream of the *Lipin* start codon. A protospacer of the sequence 5'-CAGACCAAAGATGAATAGCC-3' was cloned into pCFD3 (Addgene) and the resulting guide RNA expression vector injected into embryos expressing Cas9 in the germline (Bestgene Inc.; yw;;nos-Cas9(III-attP2)). Frame-shift mutations resulting from erroneous non-homologous end joining were identified by screening flies carrying mutagenized chromosomes by PCR and DNA sequencing.

A C-to-G nucleotide exchange leading to an amino acid exchange (D812E) in the catalytic motif of Lipin (*Lipin*^{D812E}) was introduced by ends-in gene targeting (19). A 6-kb fragment of the *Lipin* gene was amplified from Bac-Clone #RP98-9N11 (BACPAC Resources Center) using PCR primers 5'-GCTGCGGCCGCGTTGCTATGGCTGTGGCCAC-3' and 5'-GACTGGGTACCCACCAGCGCCGTCTCCAGCTC-3' and cloned into the *Kpn*I and *Not*I sites of pBluescriptKSII. The C-to-G nucleotide exchange was then introduced using primers of the sequence 5'-GGTGGTGATCTCGGAGATTGACGGCACCATCA-3' and 5'-GCCATTCAGCCGTACGACTAGGTTAGGC-3' using a Change-IT Multiple Mutation Site Directed Mutagenesis Kit (Affymetrix/USB; Santa Clara, CA, USA). A recognition site for

the I-SceI homing meganuclease was introduced by PCR using primers 5'-CATCGAACCAGGTATTACCCAGTTATCCCTAGGCGGTCGAACTCCTCGTCCGAGGGTGGT-3' and 5'-GCTGCGGCCGCGTTGCTATGGCTGTGGCCAC-3'. The resulting product was cut at *Not*I and *Sex*AI sites introduced through the primers at the 5'- and 3'-ends, respectively, and used to replace the corresponding fragment in the original mutagenized pBluescript construct. Finally, the modified *Lipin* DNA was excised from the vector and ligated into the *Not*I and *Kpn*I sites of targeting vector P[TV2] to create a donor construct for injection into *Drosophila* embryos (Bestgene Inc.). A mutant stock was established following the published protocol for ends-in targeting (19).

Rescue experiments

To determine if *Lipin* null mutants can be efficiently rescued by fat body or ubiquitous expression of wild-type *Lipin*, we carried out crosses to create animals of the genotypes *Lipin^{KO}/Df(2R)Exel7095; r4-GAL4/UAS-LipinWT* and *Lipin^{KO}/Df(2R)Exel7095; tub-GAL4/UAS-LipinWT*. Survival of these animals was compared to sibling control animals from the same vials carrying one copy of the *Lipin* wild-type allele plus GAL4 driver and UAS-responder or one copy of the *Lipin* wild-type allele plus GAL4 driver or UAS-responder. Pupae were collected daily and genotyped. Further development was monitored daily. Eclosing adults were isolated and crossed to flies of the opposite sex to determine fertility. To determine whether *r4-GAL4* expression was indeed specific to the fat body, as reported (18), we crossed *r4-GAL4* flies with flies carrying *UAS-2xeGFP*. Fluorescent microscopy confirmed strong expression in the larval and adult fat bodies that was maintained until at least five weeks after eclosion. In addition, GFP expression

was observed in the adult hindgut and Malpighian tubules. Thus, tissue specificity of the *r4-GAL4* driver is not quite as strict as assumed in previous studies.

Lipin immunostaining, starvation, and longevity experiments

Larvae of the genotypes *Lipin$^{\Delta NLS}$/Df(2R)Exel7095* and *Lipin/Df(2R)Exel7095* were reared on standard food. Feeding third instar larvae were removed from the food, transferred to 1% agar starvation plates and kept in an incubator at 25°C for about 16 hours. Fat bodies dissected from the starved larvae and fed control larvae were stained with an affinity-purified antibody directed against Lipin and DAPI to visualize DNA as previously described (12).

To examine starvation resistance of *Lipin$^{\Delta NLS}$/Df(2R)Exel7095* and control flies carrying the wild-type *Lipin* allele of the injection stock over *Df(2R)Exel7095*, newly eclosed flies were collected over 24 hours and kept for three days on standard food. Flies were then separated by sex and groups of 25 flies transferred into starvation vials with water-saturated Fly Plugs at the bottom (Genesee Scientific). Survival was monitored daily and dead flies removed. Flies were regularly transferred to fresh vials with water-saturated plugs to avoid feeding on mold or, in the case of the females, eggs deposited onto the plugs. Experiments were carried out in biological triplicates.

To compare the longevity of *Lipin$^{\Delta NLS}$/Df(2R)Exel7095* flies to the longevity of control flies, we proceeded as for the starvation experiments, but kept flies on standard food

instead of starving them. Flies were transferred to fresh food every 3-4 days and survival was monitored daily. Experiments were carried out in biological triplicates.

Triglyceride assays and lipid staining

TAG was measured using a colorimetric assay (20). Briefly, flies were collected as for the starvation assays and samples were obtained by pooling and homogenizing 10 male or 7 female flies of the same genotype in 500 µL PBS containing 0.05% Tween 20 (PBST). After setting aside aliquots for protein measurements (Bradford Protein Assay Kit #23200, Thermo Scientific), homogenates were heated to 70°C for 10 min to inactivate enzymes released from the cells. For measurement of free glycerol, 80 µL of each sample were mixed with PBST and, for measurement of glycerol released from TAG, another 80 µL with Triglyceride Reagent (Sigma T2449). The samples were then incubated at 37°C for 1 hour. After mixing 150 µL of sample with 600 µL of Free Glycerol Reagent (Sigma F6428) and incubation at 37°C for 5 min, absorbances were measured at 540 nm using polystyrene standard cuvettes and compared to a standard curve derived from Glycerol Standard solutions (Sigma G7793). Means for males shown in Fig. 4 were derived from 7 (*Lipin*$^{\Delta NLS}$) or 9 (control) biological replicates, means for females from 3 (*Lipin*$^{\Delta NLS}$) or 4 (control) biological replicates.

Fat body from wandering third instar larvae was stained with Bodipy 493/503 (Invitrogen/Molecular Probes) to visualize fat droplets as described previously (12).

RNA-seq

For RNA-seq analyses, freshly eclosed male and female flies of the genotype *Lipin*$^{\Delta NLS}$*/Df(2R)Exel7095* and control flies of the genotype *Lipin/Df(2R)Exel7095*, carrying the wild-type *Lipin* allele of the CRISPR/Cas9 injection stock, were collected and kept on regular food for three days. Male and female flies were then separated and placed in groups of 15 and 10, respectively, in food-containing vials or starvation vials containing only a source of water. After 24 hours under continued feeding or fasting conditions the flies were processed for RNA-seq. RNA was extracted from three biological replicates for each combination of genotype, sex, and condition (fed, starved). Flies were snap-frozen in liquid nitrogen and transferred into Trizol Reagent (Invitrogen Life Sciences) for homogenization and RNA extraction. RNA was further purified using a Qiagen RNeasy Mini Kit and its quality determined using an Agilent TapeStation. Oligo-dT based library preparation and RNA sequencing were carried out at the Functional Genomics Facility of the University of Chicago. This was followed by bioinformatics analysis at the University of Chicago's Center for Research Informatics. Illumina RNA sequencing was done using a NovaSEQ 6000 sequencer performing 60M 100-bp paired-end reads (30M clusters) per sample.

After data normalization, differential expression analyses were performed using the R/Bioconductor software package limma (21). Reads were aligned to the *Drosophila melanogaster* assembly BDGP6.22 (Ensembl release 98). The following eight comparisons were made (Supplementary Tables S4-S11): wild type males, fed vs starved; wild-type males, fed vs mutant males, fed; wild-type males, starved vs mutant

males, starved; mutant males, fed vs mutant males, starved; wild type females, fed vs starved; wild-type females, fed vs mutant females, fed; wild-type females, starved vs mutant females, starved; mutant females, fed vs mutant females, starved. Differentially expressed genes were identified by controlling their Benjamini-Hochberg adjusted p-values under false discovery rate cutoff 0.05 and base 2 log fold-change of 1.5. Overrepresentation among differentially expressed genes of gene ontology (GO) terms was analyzed using the Bioconductor clusterProfiler package (22). If not indicated otherwise, tissue-specific gene expression data presented in Tables 1, 2, S1, S2, and S3 were derived from the *Drosophila* gene expression atlases FlyAtlas 1 and 2 (23, 24).

Metabolic rate measurements

Metabolic rate was measured as volume CO_2 (VCO_2; µL/min) produced per fly using open-flow respirometry. A minimum of seven independent fly cohorts were measured for each genotype and sex. Flies were kept on standard food for one to two weeks after eclosure and then separated by sex into groups of seven flies that were transferred to fresh standard food. Flies were allowed to recover from the CO_2 anesthesia for 24 hours prior to transfer to respirometry chambers (25). Respirometry chambers were made by modifying 5.0 mL plastic Luer lock syringe barrels with plastic mesh and glass wool to prevent animal escape. The resulting headspace of each chamber was 1.25 mL. A total of eight chambers (one baseline and seven animal chambers) were measured during each respirometry run. All measurements were performed at the same time of the day at 25°C in the dark. During measurements, CO_2 and water free air was pushed through each chamber at a flow rate of 50 mL/min. A multiplexer (Sable Systems, Las Vegas,

NV) was used to divert the excurrent air from each chamber to a LI-COR CO_2 analyzer (LI-COR, Lincoln, NE) for measuring CO_2 (ppm) production. During each respirometry run, each chamber of seven flies was measured for 7.5 minutes per hour; baseline was measured for 3.75 min at the top and bottom of every hour. Each set of flies were measured for a minimum of six hours. Expedata (Sable Systems) was used to control the multiplexer and record data every five seconds. Expedata was also used to run baseline corrections, calculate VCO_2 per fly (μL/min), and filter data through the use of a nadir function (26). The nadir function selected the lowest continuous values of VCO_2 for a span of 50 sec per chamber per hour. The resulting filtered data was analyzed in R using the car package. CO_2 production by the flies was stable for at least four hours of measurement. The values for the first hour of measurements were used for statistical analysis, as they were the lowest recorded. CO_2 production was compared between strains and sexes using a two-way ANOVA. In addition to the significant difference detected between strains ($p=0.021$; Fig. 5), metabolic rates were also significantly different between sexes ($p=0.023$).

RESULTS

Lipin is an essential gene with vital functions outside the fat body

We used CRISPR/Cas9 mutagenesis to create a *bona fide* null allele of *Lipin*, $Lipin^{KO}$. $Lipin^{KO}$ carries a frame-shift mutation immediately after the start codon, introducing an early stop codon and resulting in a nucleotide sequence encoding a 29-amino acid random peptide (Fig. 1A). Consistent with the prediction that $Lipin^{KO}$ is a null allele, animals homozygous for $Lipin^{KO}$, or carrying the allele over the deficiency *Df(2R)Exel7095* that uncovers the *Lipin* locus, are not viable as indicated by the absence of homozygous larvae, pupae or adults. Having this mutant available, we asked whether *Lipin* is required for viability in tissues other than the fat body. Such a requirement is suggested by the broad expression pattern of *Lipin*, which is not only expressed in the fat body but also in many other tissues, including the gut, the Malpighian tubules, the brain, and the endocrine ring gland (17). We used the *r4-GAL4* driver to express wild-type *Lipin* in the larval fat body in a $Lipin^{KO}$ mutant background. Animals carrying this driver exhibit robust GAL4 expression in the larval and adult fat bodies as well as expression in the adult hindgut and Malpighian tubules (see Material and Methods). For comparison, we attempted a rescue by ubiquitous expression of a wild-type *Lipin* transgene driven by the *tub-GAL4* driver. Expression of *Lipin* driven by both the *r4* and *tub-GAL4* driver was sufficient to rescue $Lipin^{KO}$ mutants to adulthood. However, while the ubiquitous expression of *Lipin* led to rescue of more than half of the mutant animals to the pupal and adult stages, only 21% of the animals expressing *Lipin*

in the fat body were rescued to the pupal stage, and a mere 7% to the adult stage (Fig. 1B). Animals rescued by fat body expression, but not by ubiquitous expression, showed substantial pupal lethality (Fig. 1C). Moreover, many (53%) of the adults rescued by fat body expression died during or shortly after eclosion, whereas none of the adults rescued by ubiquitous expression died at that time. Male flies rescued by ubiquitous expression had a median lifespan of 61 days (n=18) and female flies of 47 days (n=14). The longest-lived male died after 104 days, and the longest-lived female after 66 days. In striking contrast, none of the flies that had been rescued by fat body expression, and hadn't already died at eclosion, lived for more than 14 days. Although flies rescued by ubiquitous expression appeared much healthier and longer lived, flies rescued by either ubiquitous or fat body-expression were infertile. Together, these data strongly suggest that *Lipin* has vital functions outside the fat body and that proper *Lipin* expression is particularly critical for fertility.

Lipin's PAP motif, but not its NLS, is required for the development and viability of adult flies

Next, we asked whether Lipin's PAP activity and/or nuclear functions of the protein are required for development and survival. To generate a mutant that lacks Lipin's PAP activity, we used ends-in gene targeting to introduce a single amino acid exchange into Lipin's catalytic DIDGT motif at position 812, changing it to EIDGT (19) (Fig. 1A). The D-to-E exchange in the motif leads to a complete loss of PAP activity (15). Animals homozygous for the *Lipin*^{D812E} allele developed into first instar larvae, but none of these larvae reached the second larval instar. The animals could be rescued from the lethality

by expressing wild-type *Lipin* from a transgene in the $Lipin^{D812E}/Lipin^{D812E}$ background, confirming that the observed lethality was indeed caused by the lack of PAP activity normally provided by Lipin. Most homozygous larvae (83%; n=75) were able to survive beyond the 24 hours that it normally takes to reach the next stage, but all of them died within 60 hours as first instar larvae. This clearly demonstrates that Lipin's PA phosphatase activity is required for development beyond the embryonic and early larval stages. It seems likely that it is also required for embryogenesis, because *Lipin* is a maternally expressed gene and Lipin protein can be detected in the oocyte cytoplasm (17). This suggests that maternal Lipin rescues homozygous mutants through embryogenesis.

To create a mutant that is defective in carrying out nuclear functions of Lipin, we used CRISPR/Cas9 mutagenesis to introduce an 18-bp deletion into *Lipin* that removes the protein's NLS (Fig. 1A). We opted to remove the NLS rather than introducing point mutations into the transcriptional co-regulator motif, because the latter had been shown to also interfere with enzymatic activity of the protein (15). We were successful in isolating a $Lipin^{\Delta NLS}$ allele and found that, in contrast to the $Lipin^{KO}$ and $Lipin^{D812E}$ mutants, $Lipin^{\Delta NLS}$ mutants were viable. To avoid homozygosity for other loci on the mutagenized chromosome, we used animals carrying one copy of $Lipin^{\Delta NLS}$ and control animals carrying one copy of the wild-type *Lipin* allele of the CRISPR injection stock for all phenotypic characterizations reported here. We accomplished hemizygosity by using a previously characterized deficiency chromosome, *Df(2R)Exel7095* (17). To confirm that the mutant protein is excluded from the nucleus, we examined intracellular

distribution of Lipin$^{\Delta NLS}$ and wild-type Lipin under conditions that promote nuclear translocation of Lipin. Nuclear translocation of Lipin is observed when signaling through the nutrient-sensitive TORC1 signaling pathway is attenuated (12). To accomplish this, we subjected pre-wandering third instar mutant and wild-type control larvae to starvation conditions. As expected, starvation caused nuclear translocation of Lipin in fat body of control larvae. In contrast, we did not observe nuclear translocation of Lipin$^{\Delta NLS}$ under these conditions (Fig. 2). These data imply that *Lipin$^{\Delta NLS}$* mutants are deficient in carrying out functions that Lipin normally has in the cell nucleus, including gene-regulatory functions.

Lipin$^{\Delta NLS}$ flies have an improved life expectancy

Lipin$^{\Delta NLS}$ mutant flies were not only viable, but they also did not show any obvious phenotypic deviations from wild-type flies. Although the absence of Lipin's NLS did not appear to have major detrimental effects on survival and fecundity, we decided to examine these animals more closely by asking whether they had the same life expectancy as wild-type control flies. To determine whether this was the case, we compared the survival of *Lipin$^{\Delta NLS}$/Df(2R)Exel7095* flies to that of *Lipin^{WT}/Df(2R)Exel7095* control flies (Fig. 3). Males and female flies were kept separately on standard food and deaths were monitored regularly until all flies had died. The median lifespan of both male and female *Lipin$^{\Delta NLS}$* flies was significantly increased, an effect that was most pronounced in the females with an increase of 24 days, which amounts to an impressive 34% jump in life expectancy. Females also showed a

significant increase in maximum lifespan by 14 days, whereas maximum lifespan in males was not significantly changed (Fig. 3). These data suggest that nuclear functions of Lipin are not only not required but may even be detrimental when flies are raised on a standard diet.

Lipin$^{\Delta NLS}$ flies have increased susceptibility to starvation

In stark contrast to fed flies, *Lipin*$^{\Delta NLS}$ flies subjected to starvation showed significantly decreased survival (Fig. 3). *Lipin*$^{\Delta NLS}$*/Df(2R)Exel7095* and *Lipin*^{WT}*/Df(2R)Exel7095* control flies were separated by sex and transferred to vials containing a source of water but no food. Survival was monitored daily and dead animals were removed. Both *Lipin*$^{\Delta NLS}$ males and females showed significantly reduced maximum and median lifespans. Median lifespan of males was reduced from 9 to 3 days, and for females from 7 to 6 days. Maximum lifespan for both males and females was reduced from 10 to 6 days. Almost 40% of control females were still alive on day 7 of starvation after all *Lipin*$^{\Delta NLS}$ females had died. These data suggest that Lipin's ability to enter the nucleus is of critical importance for physiological adaptation to nutrient deprivation.

Fat stores are not reduced in *Lipin*$^{\Delta NLS}$ flies

Reduced survival of *Lipin*$^{\Delta NLS}$ flies under starvation conditions could be due to reduced fat stores of these animals. Therefore, we measured triglyceride levels of mutant and control flies (Fig. 4A). TAG levels proved to be not reduced in the mutants. On the

contrary, they appeared slightly elevated, although differences were not statistically significant. Staining with the lipid dye Bodipy confirmed that the fat body of *Lipin*$^{\Delta NLS}$ larvae contained normally sized fat droplets, while indicating slightly elevated rather than decreased levels of neutral lipids. This strongly suggests that nuclear function of Lipin, but not its function in TAG synthesis, is responsible for adaptation to starvation conditions.

Genes involved in energy homeostasis, feeding behavior and the immune response are mis-regulated in fed and food-deprived *Lipin*$^{\Delta NLS}$ flies

Together, our results suggested that nuclear functions of Lipin are less important when nutrients are plentiful, but essential under conditions of nutrient deprivation. Since lipins can act as transcriptional co-regulators (14, 15), it seemed likely that nuclear Lipin brings about changes in gene expression that promote adaptation to nutrient deprivation. To test this hypothesis, we carried out RNA-seq analyses with *Lipin*$^{\Delta NLS}$ and wild-type control flies that had been kept on a standard diet or starved for 24 hours. RNA was extracted from male and female flies separately and mRNA used for library preparation.

Consistent with the observed differences in life expectancy, we found that many genes were differentially expressed in normally fed *Lipin*$^{\Delta NLS}$ and *Lipin* wild-type flies (Supplementary Tables S4 and S5). About half of the 223 genes that showed an at least 1.5-fold difference in expression in males showed reduced expression in the mutant and

the other half increased expression. In females, a similar number of genes (n= 233) were differentially expressed. However, a considerably larger number, 76% of these genes showed increased expression in the mutant. Overall, sex differences were extensive with the expression of only 43 genes changing significantly at least 1.5-fold in both males and females. When differentially expressed genes were extracted regardless of sex, genes associated with the gene annotation term fatty acid synthase activity were enriched (Tables 1 and S1). Increased expression in the mutant was found for two genes encoding fatty acid synthases, FASN2 and FASN3, and five genes encoding putative fatty acid elongases. FASN2, FASN3 and the fatty acid elongase eloF are expressed in adult fat body and oenocytes (24, 27-29). The oenocytes are hepatocyte-like cells in *Drosophila* with functions in lipid metabolism, cuticular hydrocarbon synthesis and pheromone production (30, 31). Reduced expression of FASN2, FASN3, and eloF specifically in the oenocytes has been shown to affect hydrocarbon synthesis with consequent effects on desiccation resistance and courtship behavior (27-29, 32). In addition to eloF, four other elongases were affected by Lipin that show expression in fat body and the digestive system.

Other genes associated with lipid metabolism that were mis-regulated in the *Lipin* mutant include a *trimethyllysine dioxygenase* gene, encoding an enzyme required for carnitine synthesis. Carnitine serves as a carrier molecule for the transport of long-chain fatty acids into mitochondria, a rate-limiting step in fatty acid β-oxidation. Down-regulation of this gene specifically in $Lipin^{\Delta NLS}$ males suggests that β-oxidation is negatively affected in these flies.

Taken together, the data suggest that fatty acid metabolism is shifted from β-oxidation to lipogenesis in *Lipin*$^{\Delta NLS}$ flies. Thus, when nutrients including fats are plentiful, it seems to be an important function of wild-type Lipin to suppress the *de novo* synthesis of fatty acids and to promote the use of fatty acids for energy production. At the same time, Lipin appears to impede the use of stored carbohydrates for energy production. In both males and females, one of the most strongly up-regulated genes in *Lipin*$^{\Delta NLS}$ was *target of brain insulin (tobi)*, which encodes an α-glucosidase involved in glycogenolysis (Table 1). Tobi is specifically activated when nutritional sugar is low (33).

Another group of genes that were identified as enriched in the sex-independent analysis consisted of genes encoding serine-type proteases (Supplemental Table S1). Half of these proteases that respond to Lipin are specifically expressed in the digestive system. These data suggest that Lipin, which shows both nuclear and cytoplasmic expression in the digestive system (17), plays an important role in the gut in controlling the balance of enzymes involved in the digestion of nutritional protein.

Specifically in male flies, genes differentially expressed in *Lipin*$^{\Delta NLS}$ and wild type were enriched for oxidoreductases. Six broadly expressed cytochrome P450 genes showed reduced expression in *Lipin*$^{\Delta NLS}$ and several genes annotated as encoding prolyl 4-hydroxylases, which are specifically expressed in the male accessory gland, showed increased expression (Supplementary Table S1). The latter suggests a role of Lipin in male fertility, a conclusion that is supported by our observation that rescued *Lipin*^{KO}

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mutants remain infertile. Interestingly, the expression of certain cytochrome P450 genes is also changed in females, among them *Cyp4g1*, which encodes an oenocyte-specific ω-hydroxylase that regulates triacylglycerol composition (30).

Finally, again specifically in males, genes encoding transmembrane receptors showed enrichment (Tables 1 and S1). The gene encoding one of these receptors, *methuselah-like 8* (*mthl8*), also showed strong up-regulation in females. Interestingly, *mthl8* is also up-regulated when the transcription factor Cabut is reduced, which is involved in nutrient sensing and rapidly induced by sugar feeding (34). In addition, three gustatory receptors showed altered expression in *Lipin*$^{\Delta NLS}$ males. Gustatory receptor GR64f is expressed in sugar-sensing neurons of the proboscis and functions as a co-receptor for the detection of sugars in the food (35). Sugar-sensing neurons also mediate fatty acid taste, specifically through activation of the phospholipase C pathway (36). Therefore, it is interesting to note that phospholipase C encoded by *CG14945* is altered in females. Together, these data suggest an involvement of Lipin in the control of responses to nutritional sugars and, possibly, fatty acids. A role in the control of feeding behavior is further supported by altered expression of several other genes, *tiwaz* in males, which encodes an adult feeding regulator that negatively regulates meal size (37), the *takeout* (*to*) feeding regulator in both males and females (38, 39), and the intestinal feeding regulator *hodor* in females (40) (Table 1).

The diminished starvation resistance of *Lipin*$^{\Delta NLS}$ flies suggested that genes that normally respond to starvation do not do this, or to a lesser degree, in the mutant. As expected, a large number of genes showed a significant, 1.5-fold or larger change in

expression upon starvation in both males (n = 992) and females (n = 495) (Supplementary Tables S6 and S7). Not surprisingly, gene ontology analysis revealed that metabolic genes, including genes involved in lipid and carbohydrate metabolism, were enriched among these genes. To test the prediction that the starvation response of some of the genes depended on the presence of wild-type Lipin, we filtered for genes that showed an at least 50% reduction of the starvation response in the *Lipin*$^{\Delta NLS}$ mutant. This led to the identification of 141 genes in females and 96 genes in males that showed a blunted response to starvation. Many of these genes were immune response genes or involved in energy metabolism (Tables 2 and S2).

Interestingly, *Lipin*$^{\Delta NLS}$ flies showed a blunted response of genes encoding enzymes that produce key intermediates of fatty acid metabolism, acyl-CoAs and malonyl-CoA, and that are normally up-regulated during starvation. Three acyl-CoA synthetases (ACS) involved in fatty acid activation, which normally show increased expression during starvation in females, showed no or reduced increases in the *Lipin*$^{\Delta NLS}$ mutant. One of them, pudgy (pdgy), localizes to mitochondria and fatty acid β-oxidation is reduced in *pdgy* mutants, suggesting that acyl-CoAs produced by pdgy are primarily used for energy production (41). In another study, *pdgy* mutants were shown to have decreased starvation resistance (42). Thus, at least some of the reduced starvation resistance of *Lipin*$^{\Delta NLS}$ flies may be due to the impaired activation of pdgy and the other ACS. In females, starvation also activated the gene encoding acetyl-CoA carboxylase (ACC), which catalyzes the rate limiting step in fatty acid synthesis. In males, ACC was significantly increased as well, but below the 1.5-fold threshold applied here. ACC is the

single gene encoding acetyl-CoA carboxylase in *Drosophila*. Knockdown of ACC in the fat body reduces TAG storage, which is consistent with its role in producing malonyl-CoA for the *de novo* synthesis of fatty acids (43). Malonyl-CoA is also known to block the carnitine shuttle that transports fatty acids into mitochondria for β-oxidation. Dampening of the carnitine shuttle is also suggested by Lipin-dependent down-regulation of two genes in males encoding enzymes required for carnitine synthesis, trimethyllysine dioxygenase and γ-butyrobetaine dioxygenase. This suggests that Lipin contributes to a down-regulation of the carnitine shuttle during long-term starvation in both males and females.

Another lipid metabolic gene that was down-regulated in response to starvation, but less so in *Lipin*$^{\Delta NLS}$ males, was FASN1. FASN1 is a broadly expressed fatty acid synthase that is strongly expressed in the fat body and required for the accumulation of normal TAG stores (24, 27). In addition to FASN1, the starvation response of several lipases was altered in *Lipin*$^{\Delta NLS}$ flies. Consistent with a shift from fatty acid synthesis to lipolysis, a fat body-expressed lipase (CG7367) was up-regulated in females. In males, the fat body-expressed lipase 4 was down-regulated, but lipase 4 is also strongly expressed in the digestive system, suggesting that overall down-regulation of this lipase primarily reflects the reduced requirement for digestion of nutritional fats. Conspicuously, many other genes that showed a reduced response to starvation in *Lipin*$^{\Delta NLS}$ are predominantly or exclusively expressed in the digestive system, especially the midgut (Tables 2 and S2). This suggests that Lipin regulates genes in the gut that are involved in the uptake and processing of nutrients. Among these genes is *p38c*, which encodes a

MAP kinase functioning in intestinal lipid homeostasis. Flies deficient in p38c accumulate neutral lipids in the gut, suggesting that the kinase controls lipid processing by the tissue (44). Another example is *Nplp2*, a gene that is important for dietary lipid extraction and effective lipid storage in the fat body (45).

Interestingly, our data show that the up-regulation under starvation conditions of *lipase 3* (*Lip3*), another lipase that is expressed in the digestive system, is considerably inhibited in *Lipin*$^{\Delta NLS}$ flies (Table 2). In the fed state, *Lip3* is negatively regulated by Lipin (Table 1). Lip3 is regulated by the fatty acid-activated nuclear receptor HNF4, which up-regulates *Lip3* under starvation conditions (46, 47). Thus, the reduced up-regulation of *Lip3* in *Lipin*$^{\Delta NLS}$ flies suggests that HNF4 and Lipin cooperate in *Lip3* activation. In mammals, lipin 1 directly interacts with HNF4α to regulate lipid metabolic genes (15). The *Drosophila* HNF4 ortholog has an important role during starvation when it activates genes that act in fatty acid β-oxidation and lipolysis, such as *Lip3* (47). We therefore asked whether the two proteins share additional potential target genes. HNF4 expression is not changed in the *Lipin*$^{\Delta NLS}$ mutant, neither under fed nor under starved conditions. Thus, potential responses of HNF4 target genes to Lipin should be independent of HNF4. Genes regulated by HNF4 in *Drosophila* have been identified by microarray studies using third instar larvae (47). When we compare these data with our RNA-seq data, we find that, in the fed state, HNF4 and Lipin share no target genes in females and only four genes in males. However, in the starved state, this number increases for males and females together to 22 genes, corresponding to 7% of the genes that respond to HNF4 under starvation conditions (Supplementary Table S12).

Among the shared targets under both fed and fasting conditions is the trimethyllysine dioxygenase encoded by *CG4335* and, under fed conditions, the γ-butyrobetaine dioxygenase encoded by *CG5321* and a predicted lipase (*CG6295*). The transmembrane receptor gene *mthl8*, which is up-regulated in fed $Lipin^{\Delta NLS}$ flies, is up-regulated in both $Lipin^{\Delta NLS}$ and *HNF4* mutant flies under starvation conditions as well (Supplementary Table S12).

In summary, our data reveal complex changes in the starvation response of genes involved in lipid metabolism that depend on Lipin. Overall, these changes seem to promote lipolysis while lowering fatty acid synthesis, as one would expect based on the energy demands of starved flies.

In addition to genes involved in lipid metabolism, a similar number of genes involved in carbohydrate and mitochondrial energy metabolism showed a reduced response to starvation in the $Lipin^{\Delta NLS}$ mutant. Among these are genes promoting glycogen breakdown (*Gbs-76*, *CG9485*) and sugar transport (*CG9657*, *CG17930*) that are down-regulated during starvation in males. *CG9485*, which encodes an ortholog of glycogen debranching enzyme AGL, is also down-regulated when insulin signaling is reduced in the larval fat body of *Drosophila* (48). Another gene associated with glycogen storage, *Acer*, is reduced in starved females. *Acer* mutants of *Drosophila* have reduced glycogen stores. It is, therefore, somewhat surprising that these mutants exhibit slightly increased starvation resistance (49). Thus, reduced down-regulation of *Acer* in the $Lipin^{\Delta NLS}$ mutant may contribute to the starvation sensitivity of the mutant. The up-regulation of several

genes by starvation that promote energy production by glycolysis and oxidative phosphorylation, including *6-phosphofructo-2-kinase*, *Phosphoglucose isomerase* and *klumpfuss* (50), was impaired in *Lipin*$^{\Delta NLS}$ (Table 2). Thus, combined, the data obtained for fed and fasting conditions suggest that it is the basic function of nuclear Lipin to stimulate catabolic processes and energy production.

Consistent with the observed effects of Lipin on genes controlling feeding behavior in the fed state, our data suggest that Lipin also contributes to changes in feeding behavior induced by starvation (Table 2). FIT, a satiety hormone produced by the fat body, which is essential for feeding control in females and strongly down-regulated during starvation (51), shows reduced down-regulation in the *Lipin*$^{\Delta NLS}$ mutant. Likewise, reduced expression of *Root* is blunted in the mutant, a gene that is required for the normal function of sugar-sensing neurons (52). Thus, our data support a role of Lipin in controlling feeding behavior in both fed and starved flies.

Interestingly, for some genes, the starvation response was enhanced in the *Lipin*$^{\Delta NLS}$ mutant, indicating that Lipin normally limits the starvation response of these genes. Enriched among these genes in males were, again, genes involved in lipid metabolism (Tables 2 and S3). The lipase Brummer and two other lipases were more strongly induced during starvation, suggesting that accelerated depletion of fat reserves may contribute to the reduced starvation resistance of *Lipin*$^{\Delta NLS}$ males. Interestingly, a target of the nutrient-responsive TORC1 pathway, the translational repressor 4E-BP1 (Thor), was more strongly activated in the *Lipin*$^{\Delta NLS}$ mutant. Equally noteworthy is the enhanced

down-regulation of *Gpdh1*, which encodes cytosolic glycerol-3-phosphate dehydrogenase, a key enzyme linking carbohydrate and lipid metabolism. Finally, genes encoding antimicrobial peptidoglycan-recognition proteins showed an enhanced response in the mutant, adding to the suite of immune response genes whose starvation response depends on Lipin (Supplementary Tables S2 and S3).

Metabolic rate is reduced in *Lipin$^{\Delta NLS}$* flies

The RNA-seq analyses indicated that interference with Lipin's ability to translocate into the cell nucleus has broad effects on the expression of genes involved in energy homeostasis. In particular, they suggested a shift to reduced β-oxidation in fed *Lipin$^{\Delta NLS}$* flies, which predicts that energy production by oxidative phosphorylation is reduced in these flies. To test this hypothesis, we examined whether *Lipin$^{\Delta NLS}$* and control flies exhibited differences in their metabolic rates. Metabolic rate in male and female flies was measured as CO_2 production by open-flow respirometry. We found that the metabolic rates were indeed significantly reduced by 8% in male and by 9% in female flies (Fig. 5). These results confirm our prediction that energy production is throttled in *Lipin$^{\Delta NLS}$* flies and are consistent with our observation that *Lipin$^{\Delta NLS}$* flies were longer lived and, thus, seemed healthier than control flies.

DISCUSSION

We created three different *Lipin* mutants, $Lipin^{KO}$, $Lipin^{D812E}$, and $Lipin^{\Delta NLS}$, to distinguish between requirements for different activities of the Lipin protein during development and different metabolic states. We had previously shown that animals homozygous for a hypomorphic allele of *Lipin* exhibit delayed development and late-larval and pupal lethality (17). Our data with the newly generated $Lipin^{KO}$ and $Lipin^{D812E}$ mutants now shows that zygotically expressed *Lipin* and its enzymatic activity are absolutely required for larval development beyond the L1 stage. This result is consistent with our previous observation that expression of $Lipin^{D812E}$ from a transgene does not rescue *Lipin* mutant phenotypes (12). Rescue experiments show that ubiquitous, but not fat body-restricted expression, of wild-type *Lipin* can fully rescue the *Lipin* null mutant (except for fertility), indicating that *Lipin* has essential functions outside the fat body. Flies rescued by ubiquitous expression appeared healthy and had a life expectancy that was comparable to that of wild-type flies, whereas most of the flies rescued by fat body expression died shortly after eclosion. Interestingly, however, all rescued flies remained sterile, indicating a requirement of Lipin for reproduction that is sensitive to the timing and/or amount of Lipin expression in one or more tissues. In contrast to Lipin's PAP activity, interference with the ability of the protein to translocate into the cell nucleus does not impair survival under normal feeding conditions. Animals that entirely rely on Lipin without an NLS are not only viable, but they also appear healthier and live longer than wild-type flies. This finding is consistent with our previous observation that $Lipin^{\Delta NLS}$ expressed from a transgene rescues lethality and larval fat body phenotypes of *Lipin* mutants to a similar

extent as wild-type *Lipin* (12). The improved life expectancy of *Lipin*$^{\Delta NLS}$ flies correlates with changes in the expression levels of metabolic genes. However, in stark contrast to the improved survival under fed conditions, lack of the NLS proved to be detrimental under starvation conditions. The requirement of nuclear translocation to resist starvation is consistent with earlier data showing that Lipin translocates into the cell nucleus when nutrients are scarce and activity of the TORC1 pathway is low (12).

Our RNA-seq analysis provides a first insight into the genomic response to a loss of *Lipin* function in *Drosophila*. Previous studies in yeast, mice, and *C. elegans* identified candidate genes that respond to loss of lipin (14), were based on overexpression of the protein (15), or addressed lipin-dependent changes in response to sugar-rich diet (16). Overexpression of *lipin 1* in mouse liver led to the changed expression of almost 4,000 genes (15). Among the up-regulated genes were the genes encoding nuclear receptors PPARα and HNF4α, which were also shown to physically interact with lipin 1 (15). Thus, at least some of the response to lipin 1 is likely mediated by these two transcription factors and lipin 1 in a cooperative fashion. PPAR's, which are known as key regulators of lipid and energy metabolism in mammals, have not been identified in *Drosophila*, whereas an HNF4 ortholog has. *Drosophila* HNF4 acts predominantly during starvation, activating genes involved in lipolysis and fatty acid β-oxidation. Since target genes of *Drosophila* HNF4 are similar to target genes of PPARα, it has been proposed that HNF4 may, at least in part, functionally substitute for PPARα in *Drosophila* (47). Comparison of our RNA-seq data with microarray data obtained for an *HNF4* null mutant did not indicate a substantial overlap between potential target genes of Lipin and HNF4.

However, it must be emphasized that the data were obtained with different source materials, whole third instar larvae in the case of HNF4 and adult flies in the case of Lipin. Therefore, they do not allow firm conclusions with respect to the extent of cooperation between HNF4 and Lipin in *Drosophila*, although negative results of genetic interaction studies do not support such a cooperation either (Q.C., unpublished observations). Still, the potential relationship between the two proteins warrants further investigation. Our data indicate that functions of Lipin and HNF4 may converge at some points, especially the carnitine synthesis pathway and the regulation of *Lip3*. Whereas both Lipin and HNF4 stimulate *Lip3* expression during starvation, only Lipin has a negative effect on *Lip3* in the fed state, leading to strong up-regulation of *Lip3* in $Lipin^{\Delta NLS}$ flies (Table 1). Since high levels of *Lip3* expression cause lipotoxicity (53), the effect of Lipin on *Lip3* may help protect cells from the detrimental effects of an overload of free fatty acids in the fed state.

Our RNA-seq data provide a first glimpse into the organism-wide genomic response to Lipin under feeding and fasting conditions. A large number of genes were differentially expressed in the fed state in both males and females between wild-type and $Lipin^{\Delta NLS}$ flies (total n=413). This does not come as a surprise, because Lipin shows partial nuclear localization in the fed state in some tissues, such as the gut and the brain, and preferential nuclear localization in at least one tissue, the Malpighian tubules (17). Strikingly, male and female flies showed a vastly different response to wild-type and mutant Lipin with an overlap of only about 20% of the genes that responded at least 1.5-fold in both sexes. This result is in accordance with an increasing number of studies that

find substantial differences in metabolic gene expression between the sexes (54-56). Especially in insects, with their yolk-rich eggs and extensive resource allocation for reproduction, sex-specific differences in metabolic gene expression do not come as a surprise. Despite these differences, the biological processes affected, which are summarized in figure 6, are similar in both males and females. The majority of the affected genes have functions in energy metabolism, in particular lipid and carbohydrate metabolism.

A prediction resulting from the RNA-seq data was that energy production is reduced in fed *Lipin*$^{\Delta NLS}$ flies. We confirmed this by showing that the flies indeed exhibit a lowered metabolic rate. Reductions in reactive oxygen species associated with low metabolic rates are being discussed as determining factors of aging and longevity (57, 58). Thus, the reduced metabolic rate may contribute to the observed increase in life expectancy of *Lipin*$^{\Delta NLS}$ flies. However, although it does not refute this possibility, it should be noted that *Drosophila* strains selected for longevity exhibit normal metabolic rates (59, 60).

In contrast to the fed state, the ability of Lipin to translocate into the cell nucleus clearly plays an essential role under conditions of nutrient deprivation. Both male and female *Lipin*$^{\Delta NLS}$ flies die significantly earlier under starvation conditions than control flies. The genomic response to starvation is substantially altered in *Lipin*$^{\Delta NLS}$ flies. The enhanced up- or reduced down-regulation of several lipases including Brummer in starved *Lipin*$^{\Delta NLS}$ males suggests that these flies break down fat stores more rapidly than control flies (Table 2). At the same time, the data suggest that they also exhaust glycogen

faster. Down-regulation of a gene encoding glycogen debranching enzyme is attenuated in *Lipin*$^{\Delta NLS}$ males and of *Gbs-76A*, which encodes a putative protein phosphatase 1 (PP-1) inhibitory subunit. PP-1 plays a critical role in glycogenolysis by inhibiting glycogen phosphorylase (61). Interestingly, the conserved NLIP domain of Lipin contains an HVRF motif which constitutes a binding site for the catalytic subunit of PP-1 (62). This raises the possibility that Gbs-76A contributes to the regulation of Lipin phosphorylation. Female *Lipin*$^{\Delta NLS}$ flies less efficiently down-regulate *Acer*, a gene that is involved in glycogen metabolism as well (49) and they less efficiently up-regulate glycolytic enzymes. This suggests that, similar to the males, females cannot make efficient use of energy reserves, although lipolytic enzymes are not as much affected in females as they are in males. Together with the presence of an additional energy source provided by degenerating oocytes, the latter may explain why starvation resistance is not as severely affected in females as it is in males (Fig. 3). The reduced activity predicted by the data of several acyl-CoA synthetases in starved *Lipin*$^{\Delta NLS}$ males and females, and of cytosolic glycerol-3-phosphate dehydrogenase in males, is consistent with the interpretation that *Lipin*$^{\Delta NLS}$ flies deplete energy reserves more quickly than control flies.

The transcriptional up-regulation of ACC during starvation, which is dampened in *Lipin*$^{\Delta NLS}$ females, was somewhat unexpected. ACC produces malonyl-CoA from acetyl-CoA for the *de novo* synthesis of fatty acids. Malonyl-CoA also has the property of blocking the carnitine shuttle that transports fatty acids into mitochondria for β-oxidation. Thus, up-regulation of ACC has the potential of counteracting the shift from lipogenesis

to β-oxidation that occurs during starvation. However, it is important to note that the activity of ACC is tightly regulated at the posttranslational level, in particular by AMPK (63). Moreover, malonyl-CoA produced by ACC is not only used for fatty acid synthesis, but also for protein malonylation (64). Interestingly, activity of the nutrient-sensitive TOR kinase can be reduced by malonylation, which may contribute to the inhibition of TORC1 during starvation (65). The possibility of increased inhibition of the carnitine shuttle after 24 hours of starvation, which must be considered as long-term starvation for flies, is not only suggested by up-regulation of ACC, but also by decreased expression of two enzymes acting in the carnitine synthesis pathway, trimethyllysine dioxygenase and γ-butyrobetaine dioxygenase. It is possible that after this extended period of starvation flies start to conserve energy stores for long-term survival.

An interesting and novel observation was that Lipin is involved in the control of immune response genes. Starvation-induced changes in the expression of a number of immune response genes depend on Lipin (Tables S2 and S3). Recent evidence suggests that interactions between Lipin and the immune response are dynamic. *Lipin* transcript levels decrease by 50% when an immune response is stimulated by expression of a constitutively active Toll receptor in the fat body. This decrease is associated with a reduction in triglyceride storage (66). Together, these observations further strengthen previously observed links between lipid metabolism and the immune response in *Drosophila* (67).

The improved life expectancy of normally fed flies that results from interference with nuclear translocation of Lipin is of particular interest in light of the recent finding that high levels of glucose induce nuclear translocation of *C. elegans* Lipin 1 and that decreased levels of Lipin 1 increase glucose toxicity (16). Mis-regulation in *Lipin*$^{\Delta NLS}$ flies of the α-glucosidase Tobi as well as Root and the Mthl8 receptor, which is regulated by the sugar-sensitive Cabut transcription factor, suggest a similar role of *Drosophila* Lipin in the metabolic response to glucose. These observations support the idea that nuclear translocation of lipins is sensitive to specific nutrients, and that lipins mediate or block genomic effects of these nutrients. It will be interesting to further investigate how nuclear translocation of *Drosophila* Lipin is fine-tuned in this manner and how this translates into healthy or unhealthy metabolic outcomes.

DATE AVAILABILITY STATEMENT

All data described in the manuscript are contained in the manuscript and the supplementary data files.

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FOOTNOTES

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TABLE 1 Genes with changed expression in *Lipin*$^{\Delta NLS}$ mutant in fed condition

FlyBase ID (FBgn)	Gene Name	Fold Change	Annotation
GO: Fatty Acid Synthase Activity/Fatty Acid Desaturation		Male/Female	Tissue	
0042627	FASN2	+1.8/+1.9	FB,OE	Fatty Acid Synthase 2
0040001	FASN3	+1.5/+1.3	FB,OE	Fatty Acid Synthase 3
0037762	eloF	+1.9/+1.5	FB,OE	Fatty Acid Elongase F
0051523	CG31523	+1.5/+1.4	DS,FB	Fatty Acid Elongase
0039030	CG6660	+1.8/+1.6	FB	Fatty Acid Elongase
0038983	CG5326	+1.5/+1.4	DS	Fatty Acid Elongase
0050008	CG30008	nc/+1.9	FB	Fatty Acid Elongase
0039755	CG15531	+1.6/+1.7	FB	Stearoyl-CoA 9-desaturase
Lipid Metabolism, other				
0023495	Lip3	+5.7/+3.4	MG	Lipase 3
0032264	Lip4	-1.7/ nc	DS,FB	Lipase 4
0038795	CG4335	-1.8/nc	FB	Trimethyllysine dioxygenase
0038730	CG6300	+3.3/+6.6	DS	Acyl-CoA synthetase
0034552	CG17999	nc/+1.7	HG	Acyl-CoA synthetase
0032402	CG14945	nc/-1.5	NS,MT FB	Phosphatidylinositol-specific phospholipase C

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Carbohydrate Metabolism				
0261575	tobi	+7.2/+9.5	FB,MG	α-glucosidase
GO: Serine-type Peptidase (see Table 1S)				
GO: Oxidoreductase Activity (see Table 1S)				
GO: Transmembrane Receptor Activity				
0052475	mthl8	+2.4/+5.7	DS,FB MT	Methuselah-like 8
0052255	Gr64f	-2.3/nd	PB	Gustatory receptor 64f, sugar-sensing neurons
Other				
0039298	to	-2.6/+1.4	DS	Takeout feeding regulator
0034636	tiwaz	+2.0/nc	NS	Adult feeding behavior
0039840	pHCL-2/hodor	nc/+1.8	DS	Intestinal feeding regulator

For a complete list of genes, see Table S1. DS, digestive system; FB, fat body; HG, hindgut; MG, midgut; MT, Malpighian tubule; NS, nervous system; OC, ocelli; OE, oenocyte; PB, proboscis; nc, not changed; nd, not detected

TABLE 2 Genes showing changed starvation response in the *Lipin*$^{\Delta NLS}$ mutant

FlyBase ID (FBgn)	Gene Name	Fold Change	Annotation
Genes showing blunted starvation response			
Lipid Metabolism		Males	Tissue	
0038795	CG4335	-2.2 > -1.5	FB	Trimethyllysine dioxygenase: carnitine synthesis
0030575	CG5321	-1.6 > -1.1	FB MT	γ-butyrobetaine dioxygenase; carnitine synthesis
0283427	FASN1	-1.5 > -1.2	FB	Fatty acid synthase 1
0032264	Lip4	-1.6 > -1.3	FB,DS	Lipase 4
		Females		
0040813	Nplp2	-1.7 > -1.2	FB,DS	Dietary lipid assimilation
0034552	CG17999	+1.6 > 1.0	HG	Long-chain-fatty-acid-CoA synthetase
0027601	pdgy	+1.6 > +1.1	DS	Acyl-CoA synthetase
0031703	CG12512	+1.6 > +1.2	DS,MT, FB	Long-chain-fatty-acid-CoA synthetase
0037996	CG4830	-23.4 > -9.0	MG	Long-chain-fatty-acid-CoA synthetase
0033246	ACC	+1.7 > +1.3	FB,DS	Acetyl-CoA carboxylase
0031976	CG7367	+4.0 > +1.9	FB,AH	Predicted lipase
0033999	CG8093	+18.3 > +9.5	MG	Predicted lipase

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0039471	CG6295	-8.9 > -3.9	MG	Predicted lipase
0267339	p38c	-1.8 > +1.1	DS	MAP kinase, intestinal lipid homeostasis
0033216	CG1946	-1.6 > +1.1	MG	Acylglycerol O-acyltransferase
		Males/ Females		
0023495	Lip3	+4.3 > +3.2 +31.9 > +16.2	MG	Lipase 3
Carbohydrate Metabolism		Males		
0036862	Gbs-76A	-1.5 > -1.2	FB,DS NS	Protein phosphatase 1 regulatory subunit
0035083	CG9485	-1.9 > -1.4	FB,DS	Glycogen debranching enzyme
		Females		
0016122	Acer	-1.5 > -1.1	FB,DS	Glycogen storage; starvation resistance
0027621	Pfrx	+1.6 > +1.1	FB.DS	6-phosphofructo-2-kinase; activation of glycolysis
0003074	Pgi	+1.5 > +1.2	UBI	Phosphoglucose isomerase; glycolytic enzyme
Mitochondrial Pathways		Females		
0013469	klu	+1.7 > +1.1	MG,NS	Klumpfuss transcription factor; citrate synthase activity
Proteases (see Table S2)				
Immune Response Genes (see Table S2)				

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Other		Females		
0038914	fit	-66.3 > -18.2	FB	protein-specific satiety hormone
0039152	Root	-2.9 > -1.5	NS	Sensory functions including taste

Genes showing enhanced starvation response				
Lipid Metabolism		Males	Tissue	
0036449	bmm	+2.7 < +4.0	FB,DS	Brummer lipase
0029831	CG5966	+2.1 < +3.9	FB,DS	Lipase
0039474	CG6283	+3.6 < +5.0	MG	Lipase
0038068	CG11600	-3.8 < -8.1	MAG	Lipase
0039184	CG6432	-2.5 < -4.5	FB	Acyl-CoA synthetase
0052072	Elo68alpha	-8.4 < -34.2	MG, MGS	Very long chain fatty acid elongation
0037534	CG2781	-2.2 < -2.9	FB,DS	Very long chain fatty acid elongation
0037765	CG9458	-2.0 < -2.7	FB	Very long chain fatty acid elongation
0037764	CG9459	-1.9 < -3.0	FB	Very long chain fatty acid elongation
0037763	CG16904	-1.8 < -2.4	FB	Very long chain fatty acid elongation
0050008	CG30008	-1.8 < -2.3	FB	Very long chain fatty acid elongation
0001128	Gpdh1	-1.8 < -2.3	DS,FB	Glycerol-3-phosphate dehydrogenase, cytosolic
Immune Response (see Table S3)				
Other				
0261560	Thor	+2.8 < +5.3	FB,DS MT	eIF4E-binding protein translational regulation

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0000053	Gart	+4.9 <+12.3	FB,DS	Purine synthesis

For a complete list of genes, see Tables S2 and S3. AH, adult head; DS, digestive system; FB, fat body; HG, hindgut; NS, nervous system; MAG, male accessory gland; MG, midgut; MGS, male genital system; MT, Malpighian tubule; UBI, ubiquitous

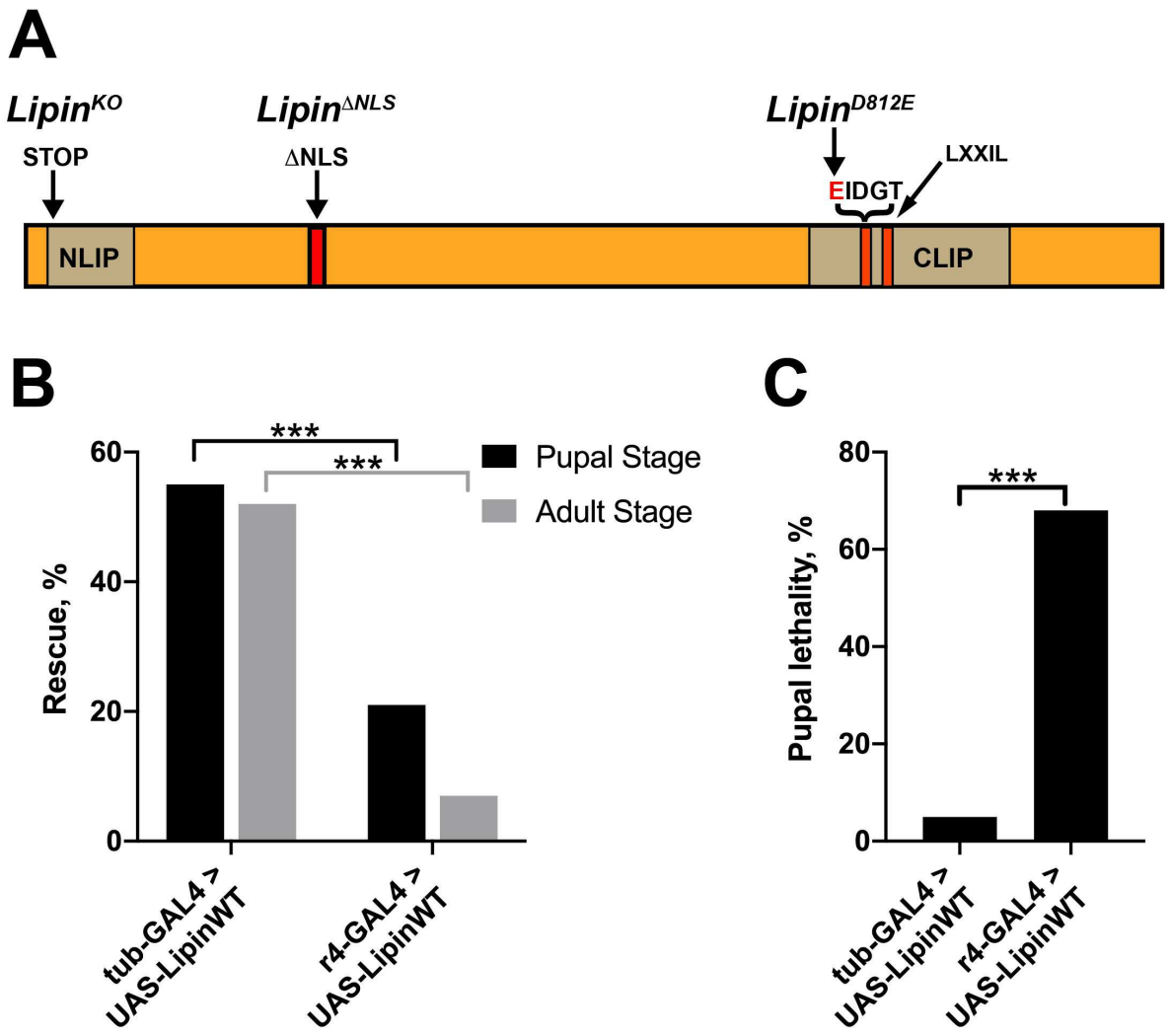

Fig. 1. (A) Structure of the *Drosophila* Lipin protein and mutations created for this study. The DIDGT motif mutated to EIDGT in *Lipin*^{D812E} signifies the catalytic motif and the LXXIL motif the transcriptional co-regulator motif. The N-terminal and C-terminal NLIP and CLIP domains are evolutionarily conserved domains shared between lipins from different organisms (4). (B) Ubiquitous expression of wild-type *Lipin* rescues *Lipin* null mutants more efficiently than expression that is largely restricted to the fat body. (C) Animals rescued by fat body-expression of *Lipin* exhibit significantly increased pupal lethality compared to animals rescued by ubiquitous expression. Wild-type *Lipin* was expressed from a *UAS-Lipin*^{WT} transgene in a *Lipin*^{KO}*/Df(2R)Exel7095* background using

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the ubiquitous *tub-GAL4* driver or the fat body *r4-GAL4* driver. *** P < 0.0001, Fisher's exact test; *tub* rescue, n=77; *r4* rescue, n=222.

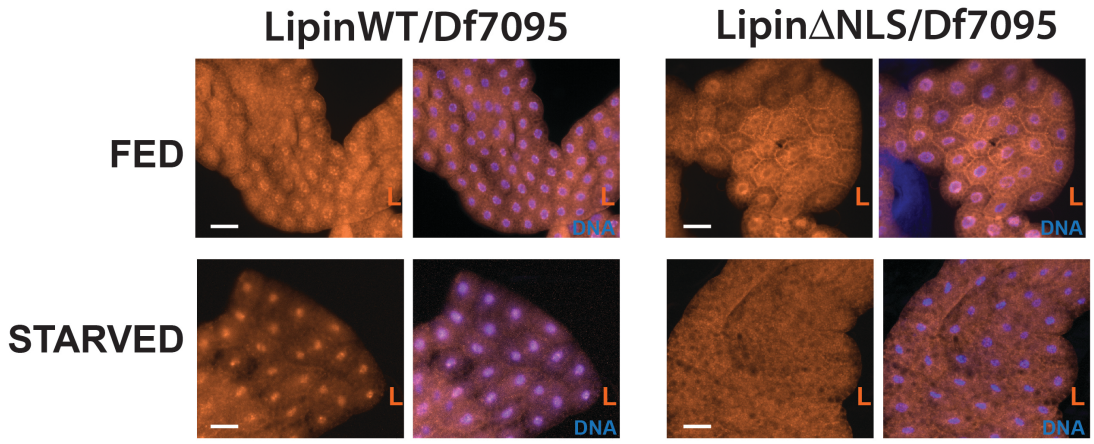

Fig. 2. Starvation causes nuclear translocation of wild-type Lipin, but not Lipin$^{\Delta NLS}$. Feeding third instar larvae of the indicated genotypes were removed from the food and subjected to starvation conditions. Fat body dissected from starved larvae and fed control larvae was stained with an antibody against Lipin (L) and DAPI to visualize DNA. Scale bars: 50 μm.

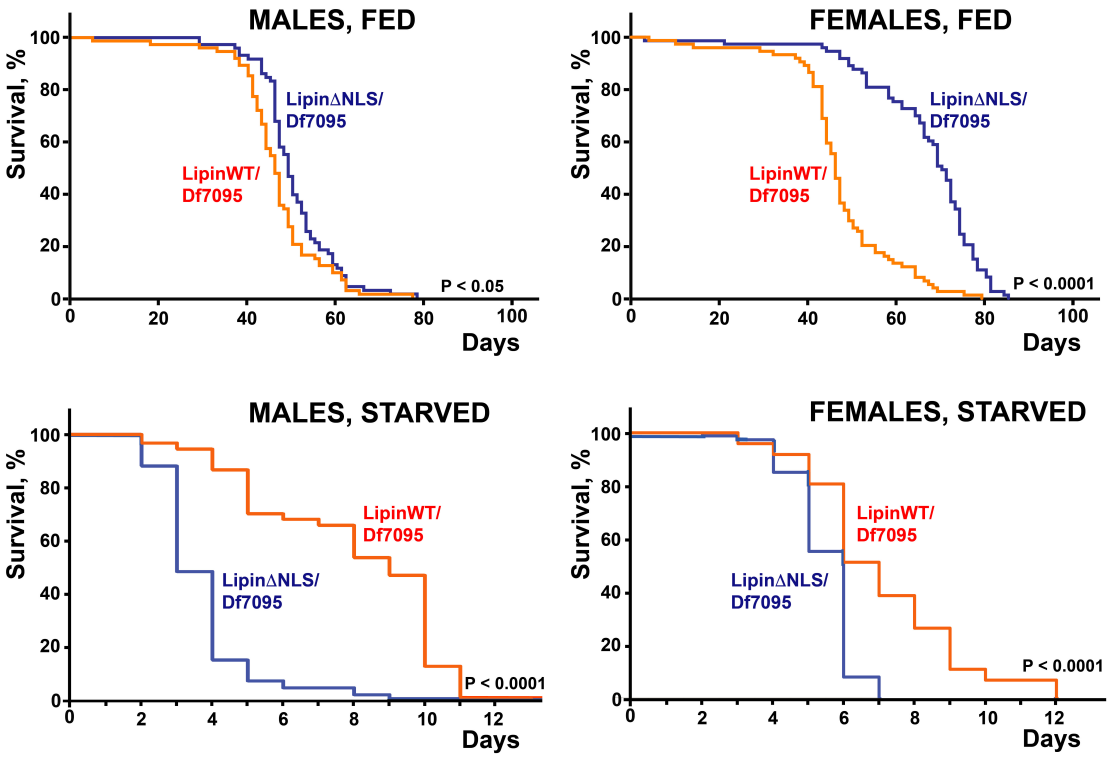

Fig. 3. Flies that rely exclusively on Lipin$^{\Delta NLS}$ have improved life expectancy but are highly susceptible to starvation. Male and female flies were kept separately on a regular or water-only diet, and survival was monitored daily. P values were determined using the log rank test.

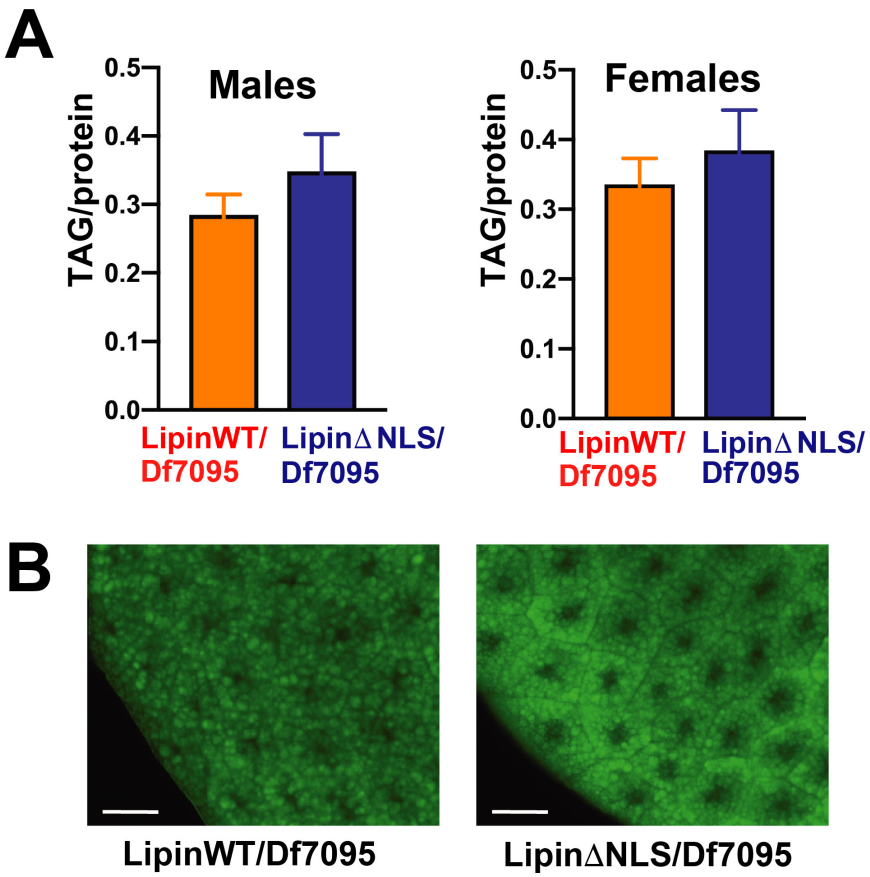

Fig. 4. *Lipin*$^{\Delta NLS}$ animals do not have reduced fat stores. (A) The ratio of total TAG to protein was determined for whole male and female flies of the indicated genotypes. Differences are not statistically significant. (B) Fat body of third-instar larvae of the indicated genotypes was stained with Bodipy to visualize fat droplets. Scale bar: 50 μm.

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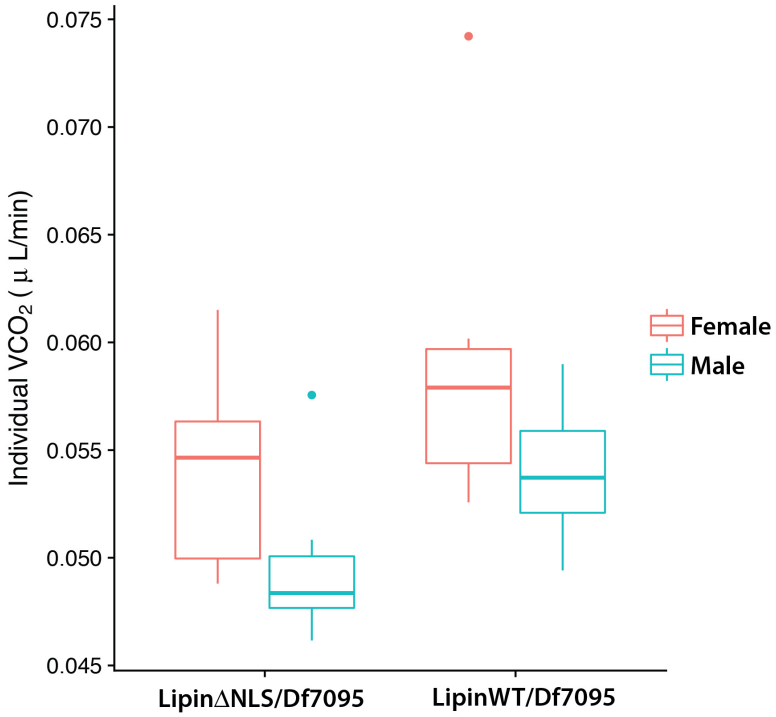

Fig. 5. Metabolic rate is decreased in *Lipin*$^{\Delta NLS}$ flies. Metabolic rate of adult *ad libitum* fed flies of the indicated genotypes was measured as CO_2 production. Differences between mutant flies and control flies were statistically significant (p=0.021; Two-way ANOVA).

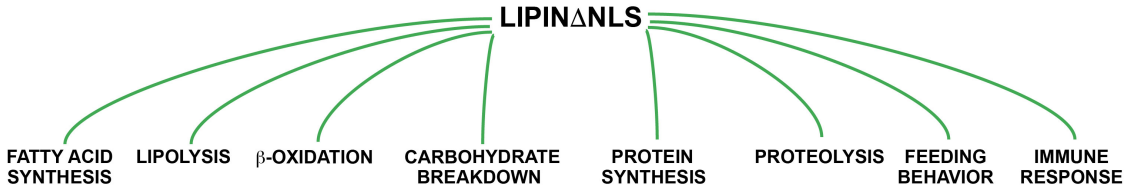

Fig. 6. Biological processes that respond to interference with Lipin's ability to migrate into the cell nucleus.